

EHMT1 Protein Binds to Nuclear Factor- κ B p50 and Represses Gene Expression^{*[S]}

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Chee-Kwee Ea[‡], ShengLi Hao[§], Kok Siong Yeo[‡], and David Baltimore^{§1}

From the [‡]Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia and the

[§]Division of Biology, California Institute of Technology, Pasadena, California 91125

Background: Methylation of histone H3 lysine 9 (H3K9) is commonly correlated with gene repression.

Results: p50 recruits EHMT1 to repress gene expression.

Conclusion: EHMT1 is a negative regulator of NF- κ B and type I interferon induction pathways.

Significance: p50-dependent changes in the chromatin structure importantly contribute to the repression of the expression genes in resting cells.

Transcriptional homeostasis relies on the balance between positive and negative regulation of gene transcription. Methylation of histone H3 lysine 9 (H3K9) is commonly correlated with gene repression. Here, we report that a euchromatic H3K9 methyltransferase, EHMT1, functions as a negative regulator in both the NF- κ B- and type I interferon-mediated gene induction pathways. EHMT1 catalyzes H3K9 methylation at promoters of NF- κ B target genes. Moreover, EHMT1 interacts with p50, and, surprisingly, p50 appears to repress the expression of type I interferon genes and genes activated by type I interferons by recruiting EHMT1 to catalyze H3K9 methylation at their promoter regions. Silencing the expression of EHMT1 by RNA interference enhances expression of a subset NF- κ B-regulated genes, augments interferon production, and augments antiviral immunity.

Covalent modifications of chromatin have emerged as key determinants of the transcriptional competence of the genome. Indeed, a strong correlation exists between the expression status of a gene and specific histone modification (1). Although histone methylation was discovered 4 decades ago (2), its physiological role was unclear until recently. Site-specific methylations of specific histone residues correlate with either activation or repression of transcription. For example, H3K4² methylation is a mark of active genes, whereas H3K9 methylation has been correlated with gene repression (3–5).

There are seven known H3K9 histone methyltransferases (H3K9-HMTs) in the human genome: SUV39H1, SUV39H2, ESET, RIZ, EHMT1 (GLP), EHMT2 (G9a), and SETDB2. The

most studied H3K9-HMTs are SUV39H1 and SUV39H2, which promote the formation of pericentric heterochromatin (6, 7). Methylation of H3K9 by SUV39H1 creates a docking site for subsequent binding of the heterochromatin protein HP1, providing compelling evidence linking H3K9 methylation to heterochromatin formation and transcriptional repression (8–10). In contrast, we know that EHMT1 and EHMT2 are the major euchromatic H3K9-HMTs because cells lacking either EHMT1 or EHMT2 show decreased H3K9me2 in euchromatin, whereas the level of H3K9me2 remains unchanged in heterochromatin (11, 12). EHMT1 is ubiquitously expressed. Knocking out the EHMT1 gene in mice leads to embryonic lethality (12). The global levels of H3K9me1 and H3K9me2, but not H3K9me3, are lower in EHMT1-deficient ES cells than in wild-type ES cells (12). However, it has been shown that EHMT1 is capable of catalyzing formation of H3K9me1, H3K9me2, and H3K9me3 in an *in vitro* methyltransferase assay (13).

NF- κ B is a transcription factor that plays a pivotal role in regulating multiple biological functions including inflammation, immunity, cell proliferation, and apoptosis. In resting cells, NF- κ B is sequestered in the cytoplasm by interaction with I κ B α . Upon activation, I κ B α is rapidly degraded by the ubiquitin-proteasome pathway. Free NF- κ B is then translocated into the nucleus where it turns on expression of a large array of target genes (14, 15). Although inducible nuclear translocation is critical for NF- κ B activation, many post-translational modifications have been shown to regulate the nuclear function of NF- κ B, including phosphorylation, ubiquitination, nitrosylation, acetylation, and methylation (16, 17).

We previously demonstrated that NF- κ B is positively regulated by methylation of the p65 subunit (17). In this report, we show that NF- κ B action as well as that of type I interferon are negatively regulated by EHMT1. We show that the expression of 44% of TNF-induced genes is enhanced in cells lacking EHMT1. Using *IL8* as a model, we further show that EHMT1 is required for establishing H3K9 methylation at the *IL8* promoter. Furthermore, EHMT1 interacts with the p50 subunit of NF- κ B. Surprisingly, p50 recruits EHMT1 to the promoters of genes that respond to type I interferon and represses the expression of these genes. Silencing the expression of either p50

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^[S] This article contains supplemental Figs. S1–S5 and Tables S1 and S2.

The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE39744).

¹ To whom correspondence should be addressed. Tel.: 626-395-8541; Fax: 626-585-9495; E-mail: baltimo@caltech.edu.

² The abbreviations used are: H3K4, histone H3 lysine 4; H3K9, histone H3 lysine 9; BMM, bone marrow-derived macrophage; HMT, histone methyltransferase; MEF, mouse embryonic fibroblast.

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or EHMT1 augments interferon production and strengthens the interferon-mediated inhibition of virus replication.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa, Human embryonic kidney (HEK) 293, HEK293-PB1, A549-PB1, wild-type, and p50-deficient mouse embryonic fibroblast (MEF) cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), penicillin G (100 μ g/ml), and streptomycin (100 μ g/ml). Bone marrow-derived macrophages (BMMs) were cultured as described earlier (18).

Antibodies—Antibodies against p65 (F6, C20), I κ B α (C21), p50 (H119, C19), JNK (C17), p38 (H147), HDAC1 (H-51), and pol II (N20) were purchased from Santa Cruz Biotechnology. Antibodies against FLAG (M2, Sigma), HA (Covance), p-JNK (Cell Signaling), p-p38 (Cell Signaling), histone H3 (Abcam, ab1791), H3K9me1 (Abcam, ab8896), H3K9me2 (Abcam, ab1220), H3K9me3 (Abcam, ab8898), and EHMT1 (R&D Systems, Bethyl Laboratories) were purchased from the respective commercial sources.

Flow Cytometry—A549-PB1 and 293T-PB1 cells were infected with influenza virus for 12 h. Cells were washed and fixed with paraformaldehyde (1% final). Fixed cells were assayed using a BD FACScan flow cytometer (BD Biosciences) and further analyzed with FlowJo software.

RT-PCR—Cells treated with TNF α for 2 h were lysed with TRIzol LS reagent (Invitrogen) to isolate total RNAs. cDNAs were synthesized with the SuperScript[®] III Reverse Transcriptase kit (Invitrogen). Quantitative PCR was performed with the 2 \times SYBR Green PCR Master Mix (Applied Biosystems) and run on the Applied Biosystems ABI 7300 Real-time PCR System. All data were normalized to L32. The sequences of the primers are listed in supplemental Table S2.

RNA Interference—All siRNAs were purchased from Santa Cruz Biotechnology: control (sc-37007), EHMT1a (sc-62261), EHMT1b (Invitrogen HSS129760), ESET (sc-45659), RIZ (sc-106513), SUV39H1 (sc-38463), SUV39H2 (sc-97240), SETDB2 (sc-62429), and p50 (sc-29407). The siRNA were transfected into HEK293 or HeLa cells by calcium phosphate precipitation at a final concentration of 10 nM. This procedure was repeated the 2nd day to increase the efficiency of gene silencing. On the 3rd day, cells were serum-starved for 18 h before treating cells with or without TNF α .

ChIP Assay—ChIP assay was carried out using the Fast-ChIP protocol with a slight modification (19). Briefly, cells were cross-linked with 1% formaldehyde at room temperature for 10 min. Excess formaldehyde was neutralized by adding 1 ml of 1.25 M glycine at room temperature for 5 min. Cells were washed three times with PBS and lysed in the hypotonic buffer. The nuclei were isolated by centrifugation at 500 \times g 4 $^{\circ}$ C for 5 min and resuspended in 0.5 ml of SDS lysis buffer (50 mM Tris, pH 8.0, 10 mM EDTA, and 1% SDS). DNAs were sheared by sonication with a Branson 450 sonicator (output 2, 90% duty cycle, 10 \times 10-s pulse). Ten micrograms of sheared DNAs was diluted with a 9 \times volume of dilution buffer (16.7 mM Tris, pH 8.0, 167 mM NaCl, 1.2 mM EDTA, 1% Triton X-100, 100 μ g/ml salmon sperm DNA, and 2% BSA). Antibodies and Dynabeads protein A (Invitrogen) were added and incubated at 4 $^{\circ}$ C for 12 h. The beads were then washed two times with a low salt

washing buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 1% Triton X-100, 2 mM EDTA, 0.5% Nonidet P-40) followed by two times with a high salt washing buffer (50 mM HEPES, pH 7.9, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% 2,5-dimethoxy-4-chloramphetamine) and one time with TE buffer. The beads were then resuspended with 100 μ l of 10% Chelex 100 beads, and cross-linking was reversed by boiling the beads for 10 min. The beads were then treated with 1 μ l of proteinase K and incubated at 56 $^{\circ}$ C for 30 min. The proteinase K was inactivated by boiling for 10 min. Supernatants containing DNA were collected, and the beads were washed with 120 μ l of dH₂O. Supernatants were collected and analyzed by quantitative PCR. The sequences of the primers are listed in supplemental Table S2.

Immunoprecipitation—For immunoprecipitation of EHMT1, HEK293 cells were transfected with FLAG-tagged p65 or p50 along with HA-EHMT1. Twenty-four hours later, the cells were lysed in radioimmune precipitation assay buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% 2,5-dimethoxy-4-chloramphetamine, 0.1% SDS, 0.5 mM DTT, 0.5 mM PMSF, and 1 \times protease inhibitor mixture (Roche Applied Science)). Two hundred micrograms of lysates was incubated with 8 μ l of FLAG-m2 beads (Sigma) or HA beads (F7, Santa Cruz Biotechnology) at 4 $^{\circ}$ C for 1 h. The beads were washed three times with radioimmune precipitation assay buffer. Bound proteins were examined by immunoblotting with FLAG or HA antibodies.

For immunoprecipitation of endogenous p65, p50, or EHMT1, HEK293 cells were treated with TNF α (10 ng/ml) and harvested at different time points. The cytoplasmic extracts were prepared using a hypotonic lysis buffer containing 0.2% Nonidet P-40 (10 mM Tris, pH 7.5, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, and 1 \times protease inhibitor mixture). The nuclear extracts were prepared by resuspending nuclei in micrococcal nuclease digestion buffer (50 mM Tris, pH 7.4, 1 mM CaCl₂, 25 mM KCl, 4 mM MgCl₂, 12.5% glycerol, 0.5 mM DTT, 0.5 mM PMSF, and 1 \times protease inhibitor mixture). The nuclear extracts were digested with MNase (90 units) at 37 $^{\circ}$ C for 5 min. The reaction was stopped by adding 1 volume of 2 \times stop solution (50 mM Tris, pH 7.4, 200 mM NaCl, 2 mM EDTA, 1% Triton X-100). The nuclear extracts were precleared by incubating with normal mouse or rabbit IgG and protein A/G-agarose beads at 4 $^{\circ}$ C for 2 h. Endogenous p65, p50, or EHMT1 was immunoprecipitated with 20 μ l of agarose-conjugated anti-p65 (F6), anti-p50 (H119), or EHMT1 (R&D) antibodies. After mixing end-to-end at 4 $^{\circ}$ C for 2 h, the beads were washed three times with PBS. Associated proteins were analyzed by immunoblotting with different antibodies as indicated.

Microarray—For microarray hybridization, 2 μ g of RNA was labeled, fragmented, and hybridized to an Affymetrix GeneChip Human Genome U133 Plus 2.0 Array. Data were then analyzed with the Rosetta Resolver gene expression data analysis system (Rosetta Inpharmatics). Expression differences \geq 2-fold with a p value <0.01 were considered significant. The Gene Expression Omnibus accession number is GSE39744.

Statistical Analysis—Data were analyzed by Microsoft Excel and are presented as the mean \pm S.D. Data are representative of three or more independent experiments. Student's t test was used to determine significant differences between groups.

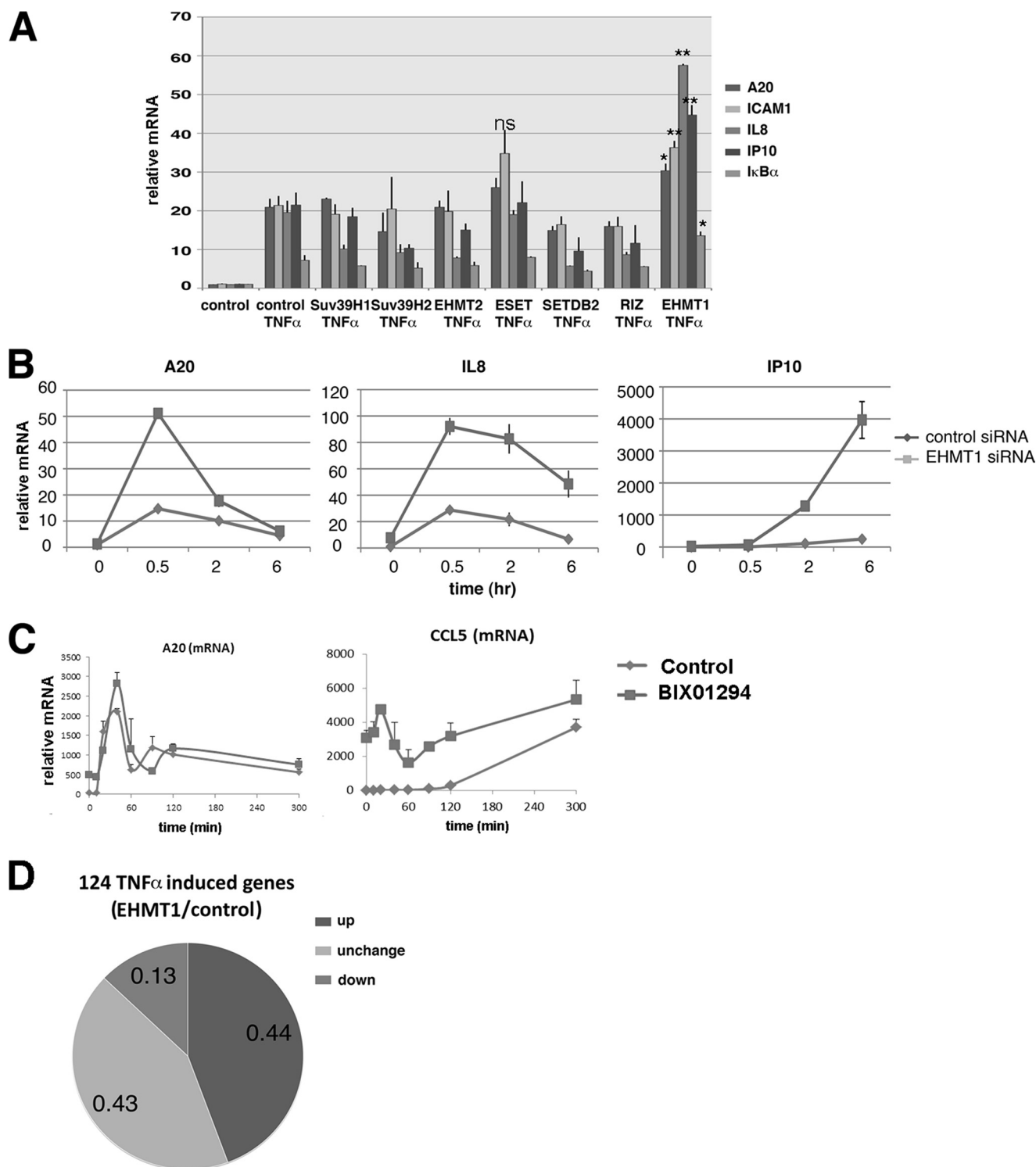


FIGURE 1. EHMT1 negatively regulates the NF- κ B pathway. *A*, HeLa cells transfected with control or H3K9-HMTs-specific siRNAs were stimulated with TNF α for 2 h. The expression of A20, I κ B α , IL8, ICAM1, and IP-10 was measured by RT-PCR (*, $p < 0.05$; **, $p < 0.01$; ns, not significant). *B*, HeLa cells transfected with control or EHMT1 siRNAs were treated with or without TNF α for the indicated time periods. The expression of A20, IL8, and IP10 was measured by RT-PCR. *C*, BMMs were treated with or without BIX01294 for 24 h then treated with or without TNF α for the indicated time periods. The expression of A20 and CCL5 was measured by RT-PCR. *D*, pie chart represents comparison of 124 TNF α -induced genes between TNF α -stimulated HeLa cells transfected with control or EHMT1 siRNAs. *A–C*, error bars represent the variation range of duplicate experiments.

RESULTS

EHMT1 Is an H3K9-HMT That Negatively Regulates the NF- κ B Pathway—To test whether H3K9 methylation regulates NF- κ B-dependent gene expression, we silenced the expression

of every known H3K9-HMT in cells using RNA interference (RNAi). Silencing the expression of EHMT1 but not other H3K9-HMTs greatly enhanced TNF α -induced expression of several NF- κ B-dependent genes including A20, ICAM1, IL8,

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IP10, and *IκBα* (Fig. 1A) (20). The efficiency of the RNAi was measured by RT-PCR, and 77–95% of knockdown efficiency was achieved (supplemental Fig. S1A). Although knocking down the expression of EHMT1 enhanced the expression of this suite of genes, it did not affect their expression kinetics (Fig. 1B). For example, mRNA for the immediate early gene, *A20*, was rapidly induced (peaked at 30 min) and then degraded, whereas that of the late gene, *IP10*, was not detected until 2 h of TNF stimulation and was further increased to 6 h, regardless of the presence or absence of EHMT1. Similar results were obtained when we treated mouse BMMs with BIX01294, a specific inhibitor of EHMT1 and EHMT2, a closely related H3K9-HMT (13, 21). The inhibition of EHMT1 in BMM led to enhanced expression of *A20* and *CCL5* in response to TNF stimulation (Fig. 1C). These results indicate that EHMT1 is the major H3K9-HMT that negatively regulates the NF-κB pathway.

To minimize the possibility that the effect we observed with EHMT1 RNAi was caused by an off-target effect, we tested two different siRNAs specific for EHMT1 (a and b). We found that TNFα-induced *IL8* expression was enhanced in cells transfected with either EHMT1a or EHMT1b siRNAs, and 70–75% knockdown efficiency was achieved with each (supplemental Fig. S1B).

We next determined whether EHMT1 regulated NF-κB activity when it was induced by IL-1β. As with TNFα stimulation, silencing the expression of EHMT1 greatly enhanced the expression of *A20*, *IP10*, and *IL8* induced by IL-1β stimulation (supplemental Fig. S1C), suggesting that EHMT1 negatively regulates NF-κB activity induced by various forms of stimulation. We further showed that depletion of EHMT1 blocked neither degradation of *IκBα* nor nuclear translocation of p65 (supplemental Fig. S2A). In addition, TNF-induced activation of JNK and p38 was also normal in EHMT1 siRNA-treated cells (supplemental Fig. S2B) suggesting that the hyperresponsiveness of EHMT1-deficient cells is not due to increased receptor-proximal signaling.

To examine the range of genes whose transcription is affected by EHMT1, we evaluated the effects of EHMT1 loss of function on TNFα-induced gene expression by global gene expression profiling. In response to TNFα stimulation, 124 genes were up-regulated (>2-fold) in control siRNA-treated HeLa cells. Treatment with EHMT1-specific siRNA further enhanced the levels of 55 genes (44%) (>1.5-fold) whereas 53 genes (43%) were not affected to 1.5-fold, and the levels of 16 genes (13%) were reduced (Fig. 1C and supplemental Table S1). It is evident that EHMT1 blockade augments transcription of many genes in response to TNFα induction.

EHMT1 Catalyzes H3K9 Methylation at the Promoters of NF-κB-regulated Genes—EHMT1 is a H3K9-HMT. In EHMT1-deficient ES cells, the global H3K9me2 but not H3K9me3 is reduced (12). Consistent with this observation, silencing the expression of EHMT1 by RNAi led to a global reduction of H3K9me2 but not of H3K9me3 (Fig. 2A). To determine whether EHMT1 catalyzes the H3K9 methylation at the *IL8* promoter, we first examined when EHMT1 is present at the *IL8* promoter by a ChIP assay. Before induction, EHMT1 was enriched at promoters of *IL8* and β-globin but not at the pro-

motor of *GAPDH* (Fig. 2B). EHMT1 was also evident in the coding region of the *IL8* gene. Knocking down the expression of EHMT1 by RNAi resulted in a 50% reduction of the ChIP signal. These results provide evidence that at the *IL8* gene, EHMT1 is present at the inactive locus before induction and reduced but not eliminated by TNF induction.

We then analyzed the levels of H3K9me2 and H3K9me3 in the control or EHMT1 siRNA-transfected cells treated with or without TNFα. In EHMT1-deficient cells, the basal levels of H3K9me2 and H3K9me3 were reduced by 40% at the *IL8* promoter and exon, but not at the β-globin promoter (Fig. 2C). This reduction was not caused by nucleosome loss because the data were normalized to the total level of histone H3. Furthermore, TNFα induced more pol II recruitment to the *IL8* gene in EHMT1-deficient cells than in control siRNA-treated cells. This is consistent with the observation that more *IL8* was produced in EHMT1-deficient cells in response to TNFα stimulation (Fig. 1A). These results suggest that EHMT1 is the major H3K9-HMT that catalyzes H3K9me2 and H3K9me3 at the *IL8* gene. Although EHMT1 is present in the β-globin promoter, it is dispensable for methylating H3K9 at the β-globin promoter.

EHMT1 Interacts with p50—We next tested whether EHMT1 interacts with NF-κB. We performed co-immunoprecipitation with lysates from HEK293 cells transfected with EHMT1 and increasing amounts of FLAG-p65 or FLAG-p50. Significantly more EHMT1 was present in FLAG-p50 immunoprecipitates than in FLAG-p65 immunoprecipitates (Fig. 3A, lanes 13–15 versus 16–18). In a reciprocal experiment, only p50, but not p65, was detected in EHMT1 immunoprecipitates (Fig. 3A, lanes 10–12 versus 7–9). These results indicate that EHMT1 interacts strongly with p50 but weakly with p65.

p50 contains two functional domains, RHD-N and RHD-C. To determine which domain of p50 is required for interacting with EHMT1, we generated p50 fragments containing either the RHD-N or RHD-C and performed co-immunoprecipitation assay as described above. We found that only the RHD-N of p50 interacted with EHMT1 (Fig. 3B, left panel). Because RHD-N of p50 contains the DNA binding domain, we further tested whether DNA binding is required for interacting with EHMT1. We generated a DNA binding-deficient mutant (p50Y60DG64E (22)) and showed that DNA binding of p50 was dispensable for the binding to EHMT1 (Fig. 3B, right panel).

In contrast, EHMT1 contains an ankyrin repeat domain, a Pre-SET, and a SET domain. To map the p50-interacting domain, serial deletion mutants of EHMT1 were generated. A fragment containing the ankyrin repeats (701–1108 amino acids) was able to interact with p50 in the co-immunoprecipitation assay (Fig. 3C), indicating that the ankyrin repeats are responsible for the interaction with p50. These findings provide further evidence that EHMT1 interacts with p50.

To test whether endogenous p50 and EHMT1 form complexes in cells, we carried out co-immunoprecipitation with nuclear extracts prepared from cells treated with TNFα for the indicated time periods. Endogenous EHMT1 was detected in p50 immunoprecipitates, regardless of TNFα treatment, but not in IgG or p65 immunoprecipitates (Fig. 3D). Interestingly, p50 was detected in TNFα-treated p65 immunoprecipitates; however, no EHMT1 was detected, suggesting that EHMT1

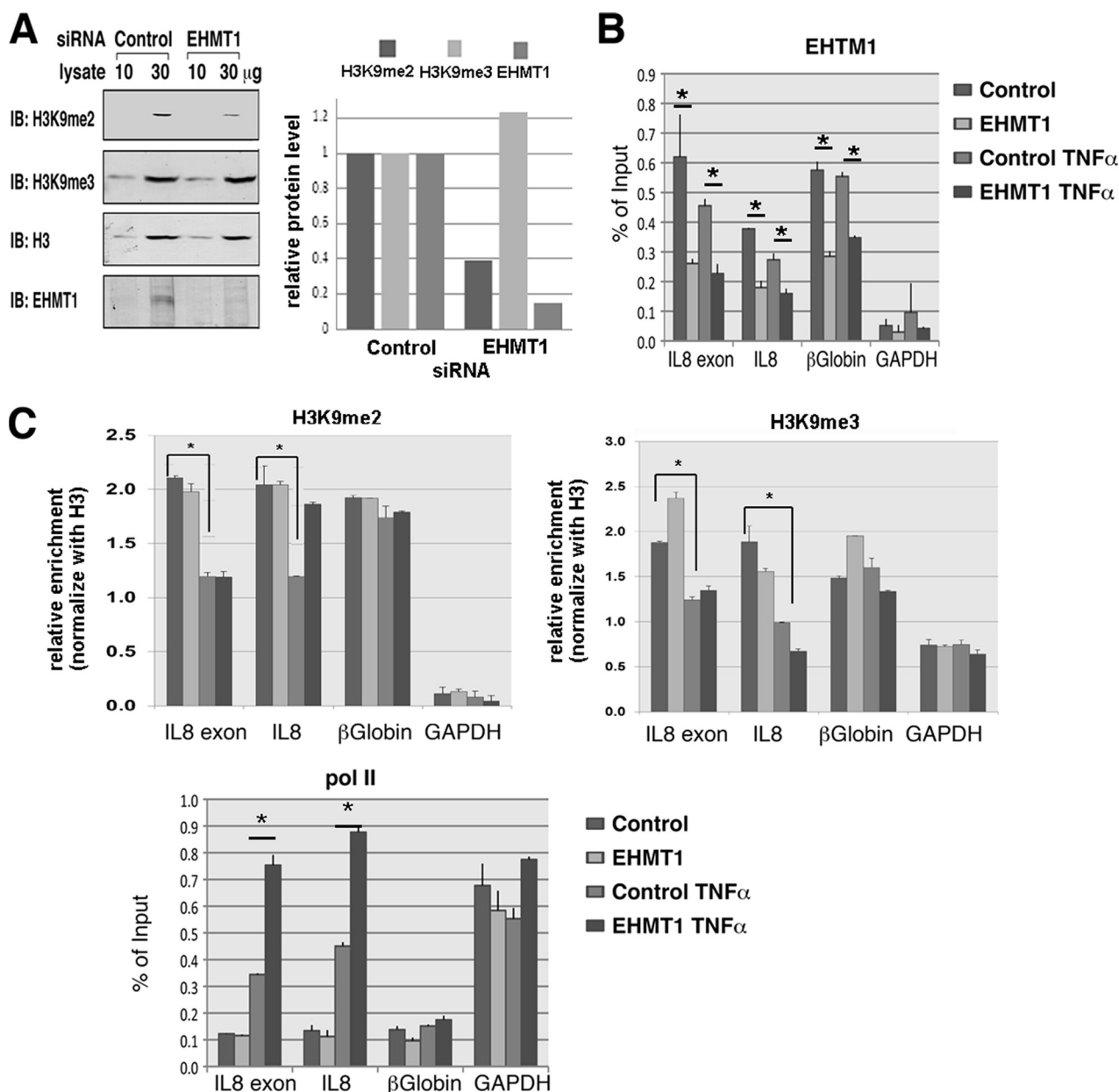


FIGURE 2. EHMT1 promotes H3K9 methylation at NF- κ B target genes. *A*, 10 or 30 μ g of nuclear extracts prepared from HeLa cells transfected with or without EHMT1-specific siRNA was immunoblotted with the indicated antibodies, the data were quantified using ImageJ, and the relative protein levels are shown (normalized with H3 protein level). *B* and *C*, HeLa cells transfected with control or EHMT1-specific siRNAs were stimulated with TNF α for 60 min and were analyzed by ChIP (EHMT1, pol II, H3K9me2/H3, and H3K9me3/H3; mean \pm S.D. (error bars); *, $p < 0.05$). Data are representative of three or more independent experiments.

interacts with p50 homodimer constitutively but not with p65-p50 heterodimer.

p50 Recruits EHMT1 to the Promoters of Type I Interferon-responsive Genes—The p50 homodimer, which lacks a transactivation domain, has been implicated in transcriptional repression (23–25). Our data show that EHMT1 negatively regulates NF- κ B target genes and interacts with p50. This suggests that the p50 homodimer may function as a transcriptional repressor by recruiting EHMT1 to the promoter of target genes to promote H3K9 methylation. To test this hypothesis, we first looked for genes that were up-regulated in both p50 and EHMT1 knockdown cells. Surprisingly, we found that expression of many type I interferon-induced genes were up-regulated

in EHMT1 knockdown cells (Fig. 4A). Some of these genes, including *IFN β* itself, *CCL5*, and *IFIT2* were also up-regulated in p50 knockdown cells (Fig. 4B). We further showed that the rise in steady mRNA levels of ISGs is not due to an increase in mRNA stability. We treated HeLa cells with BIX01294 for 24 h followed by addition of actinomycin D. We then measured the stability of mRNAs by RT-PCR and found that the mRNA stabilities of *IFIT2*, *IFIT3*, and *CCL5* mRNAs were similar whether cells were BIX01294-treated and not (supplemental Fig. S3A). Importantly, TNF α stimulation does not induce production of *IFN β* under normal conditions. In the absence of p50 or EHMT1, TNF α stimulation led to a 3-fold (p50-deficient) and 20-fold (EHMT1-deficient) increase of *IFN β* mRNA.

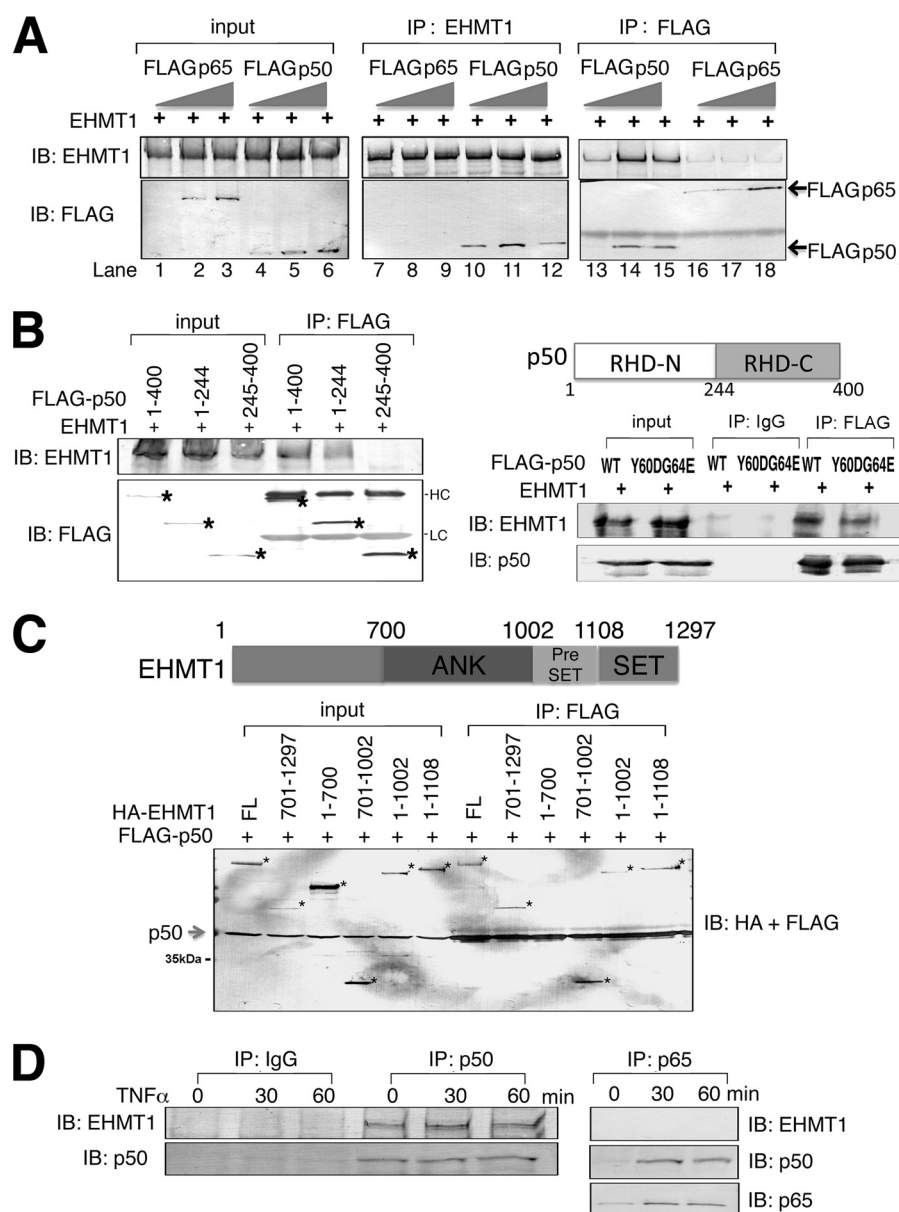
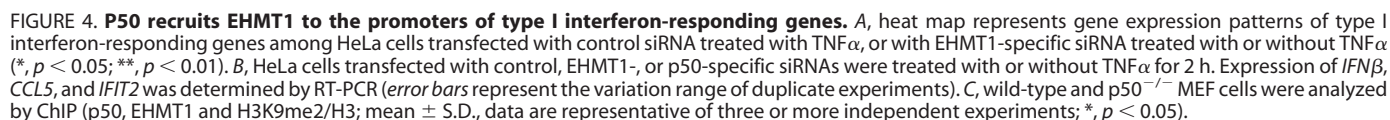


FIGURE 3. EHMT1 interacts with p50. A, HEK293 cells were transfected with EHMT1 along with increasing amounts of FLAG-p65 or FLAG-p50. EHMT1 (middle panels) and FLAG (right panels) immunoprecipitates (IP) as well as inputs (left panels) were immunoblotted (IB) with EHMT1 and FLAG antibodies. B, HEK293 cells were transfected with EHMT1 along with various fragments of p50 as indicated (left panel) or a DNA binding-defective mutant p50 (right panel). FLAG or IgG immunoprecipitates and inputs were immunoblotted with EHMT1 and p50 antibodies. Asterisks indicate different fragments of p50. HC, heavy chains; LC, light chains. C, various deletion mutants of EHMT1 along with FLAG-p50 were co-transfected into HEK293 cells. Inputs and FLAG immunoprecipitates were analyzed with HA and FLAG antibodies. Asterisks indicate different fragments of p50. D, endogenous p50 and p65 were immunoprecipitated from nuclear extracts prepared from HeLa cells treated with or without TNF α for the indicated time periods. Immunoprecipitates were probed with various antibodies as indicated. IgG immunoprecipitates were used as a negative control.

To examine this phenomenon, we determined whether p50 was present at the promoters of *IFN β* and *CCL5* by ChIP. Using wild-type and p50-deficient MEFs, we were able to detect a 3-fold increase compared with the knockout of the p50-mediated signal at the promoter of *IFN β* and *CCL5* but not at the promoter of β -globin, which only showed a background ChIP signal (Fig. 4C, top panel). In contrast, there was no enrichment of p65 at these promoters (supplemental Fig. S3B). Moreover, in the absence of p50, less EHMT1 was bound to the promoters of *CCL5* (40% decrease) and *IFN β* (50% decrease, Fig. 4C, second panel). The level of EHMT1 at the promoter of β -globin was comparable between wild-type and p50-deficient MEFs.

Consistent with the decreased level of EHMT1, the levels of H3K9me2 at the promoters of *CCL5* and *IFN β* were also decreased in the p50-deficient MEFs (Fig. 4C, and data not shown), whereas the levels of histone H3 remained unchanged (Fig. 4C, bottom panel). Similar observations were made when the expression of p50 was knocked down by RNAi in HeLa cells (supplemental Fig. S3C). The levels of EHMT1 and H3K9me2 were decreased at the promoters of *IFN β* and *CCL5* in p50-specific siRNA-treated HeLa cells. From these data, it appears that p50 represses the expression of *IFN β* and *CCL5* in response to TNF α by recruiting EHMT1, which in turn methylates H3K9.



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The ability of p50 to act as a transcriptional repressor through the recruitment of HDAC1 has been reported to be a mode of negative control of expression of some NF- κ B target genes in resting cells (26). To test whether there is cross-talk between HDAC1-mediated and EHMT1-mediated gene repression, we inhibited the activity of HDAC1 and EHMT1 with TSA and RNAi, respectively. Pretreating the cells with TSA led to higher expression of *A20* and *IL8* but not *TNF α* , *IL6*, and *IFN β* in response to TNF stimulation (supplemental Fig. S3D). Interestingly, silencing the expression of EHMT1 with RNAi further enhanced the expression of *IL8* and *A20* in the presence of TSA. Furthermore, the recruitment of HDAC1 to the promoters of *A20*, *IL8*, *TNF α* , *CCL5*, β -globin, and *IFN β* remained unchanged regardless of the presence or absence of EHMT1 (supplemental Fig. S3E). These results indicate that HDAC1-mediated and EHMT1-mediated gene repression are independent of one another.

EHMT1 and p50 Are Negative Regulators of the Type I Interferon Pathway—Viral infection of host cells triggers innate and adaptive immune responses that are essential for the survival of the host. IFN β is particularly potent as an antiviral agent. Because EHMT1 negatively regulates the expression of *IFN β* , we examined the possibility that EHMT1 could affect immune defense against influenza virus. A nonreplicative WSN strain influenza virus carrying mCherry in the place of the PB1 gene was used to infect HEK293 cells stably expressing PB1 protein (HEK293-PB1) (27). In HEK293-PB1 cells treated with a control siRNA, influenza virus infection induced the expression of *IFN β* , *CCL5*, and *IFIT2* as measured by RT-PCR (Fig. 5A). The expression of these genes was greatly enhanced in cells treated with siRNAs against p50 or EHMT1. A similar result was obtained when using p50-deficient MEF cells. Infecting the wild-type MEF cells with Sendai virus led to induction of *IFN β* , *CCL5*, and *ISG20* (Fig. 5B). The expression of these genes was further enhanced in p50-deficient MEF cells. These data confirm a negative role of EHMT1 and p50 in the type I interferon pathway.

Knocking down the expression of p50 or EHMT1 in HEK293-PB1 decreased the viral titer by 93 and 64%, respectively, compared with control siRNA-treated cells (Fig. 5C). To confirm the negative role of EHMT1 on influenza viral replication, we examined the anti-viral effect of BIX01294, a specific inhibitor of EHMT1 and EHMT2, a closely related H3K9-HMT (13, 21). We pretreated the A549-PB1 cells with BIX01294 for 48 h and infected the cells with influenza virus at a multiplicity of infection (m.o.i.) of 1. After 24 h, infected cells were quantified by sorting the virus-infected, mCherry-positive cells using FACS. Pretreating cells with BIX01294 strongly inhibited the viral infection (Fig. 5D). A similar result was observed when using vesicular stomatitis virus. Infection of HeLa cells by vesicular stomatitis virus led to nearly complete killing of the cells within 24 h at a m.o.i. of 0.05 (supplemental Fig. S4A). Pretreating cells with BIX01294 prevented cell killing by vesicular stomatitis virus even at a m.o.i. of 1. In addition, we found that the level of H3K9me2 but not H3K9me3 was reduced in BIX01294-pretreated cells, confirming the inhibition of EHMT1 by BIX01294 (supplemental Fig. S4B). Furthermore, we found that the basal level of type I interferon response genes, including

CCL5, *IFN β* , *IFIT2*, *IFIT3*, and *ISG20*, was up-regulated in BIX01294-treated cells (Fig. 5E), implying that inhibition of EHMT1 activity leads to up-regulation of antiviral genes which in turn protects cells from viral infection. These findings further support the negative role of p50 and EHMT1 in the type I interferon pathway.

DISCUSSION

We have found that the histone methylase EHMT1 is able to negatively regulate a large fraction, but not all, of NF- κ B-regulated genes. Silencing of EHMT1 causes superactivation of the genes it regulates. It does this through interaction with the p50 subunit of NF- κ B. Surprisingly, it is carried to the promoters of certain IFN-regulated genes by p50, where it exerts its negative effect on transcription. IFN β itself is one of the regulated genes so that silencing of EHMT1 or p50 augments induction of excess interferon and remarkably strengthens the inhibition of viral infection.

Our findings support a model in which p50-dependent changes in the chromatin structure of the type I interferon response genes importantly contribute to the repression of the expression of these genes in resting cells. This is consistent with the notion that p50 homodimers are involved in preinduction repression of *IFN β* gene transcription as well as subset of interferon-responding genes (28–31). The function of p50 as a transcriptional repressor involving the recruitment of HDAC1 has been reported to repress the expression of some NF- κ B target genes in resting cells (26). Here we demonstrated an alternative mechanism involving the recruitment of EHMT1 by p50 to the promoters of *IFN β* and *CCL5*. EHMT1 in turn promotes H3K9 methylation and results in the formation of chromatin structure that represses transcription (Fig. 6A). How is p50 recruited to the promoters of type I interferon response genes? STAT1 is one of the key transcription factors in the interferon pathway that regulates expression of type I interferon response genes through binding to the IREs (32). It is possible that STAT1 may play a role in recruiting p50 to the promoters of the type I interferon response genes. However, we failed to detect any interaction between p50 and STAT1 (data not shown), and a recent study has shown that p50 binds to G-rich IREs (30). Thus we think that p50 represses type I interferon response genes by directly binding to G-rich IREs. p50 has been shown to bind to some of the NF- κ B target genes in unstimulated cells (33). p50 may also recruit EHMT1 to repress the expression of a subset of TNF-induced NF- κ B target genes (Fig. 6A). In addition, EHMT1 can also inhibit the expression of NF- κ B target genes independent of p50. For example, we were unable to detect any binding of p50 at the promoters of *IL8* and *IP10* in resting cells (data not shown). The involvement of p50 in the repression of *IL8* and *IP10* is very unlikely. Thus it seems that EHMT1 is recruited in some other way to the promoters of certain NF- κ B target genes (Fig. 6B). It could involve an undetermined factor X. One strong candidate for X is the p65 subunit of NF- κ B as it has been recently shown that methylated p65 (p65K310me1) was able to repress the expression of subset NF- κ B target genes in resting cells by recruiting EHMT1 (34).

Negative regulation of transcription is an important strategy in establishing and maintaining cell- and signal-specific gene

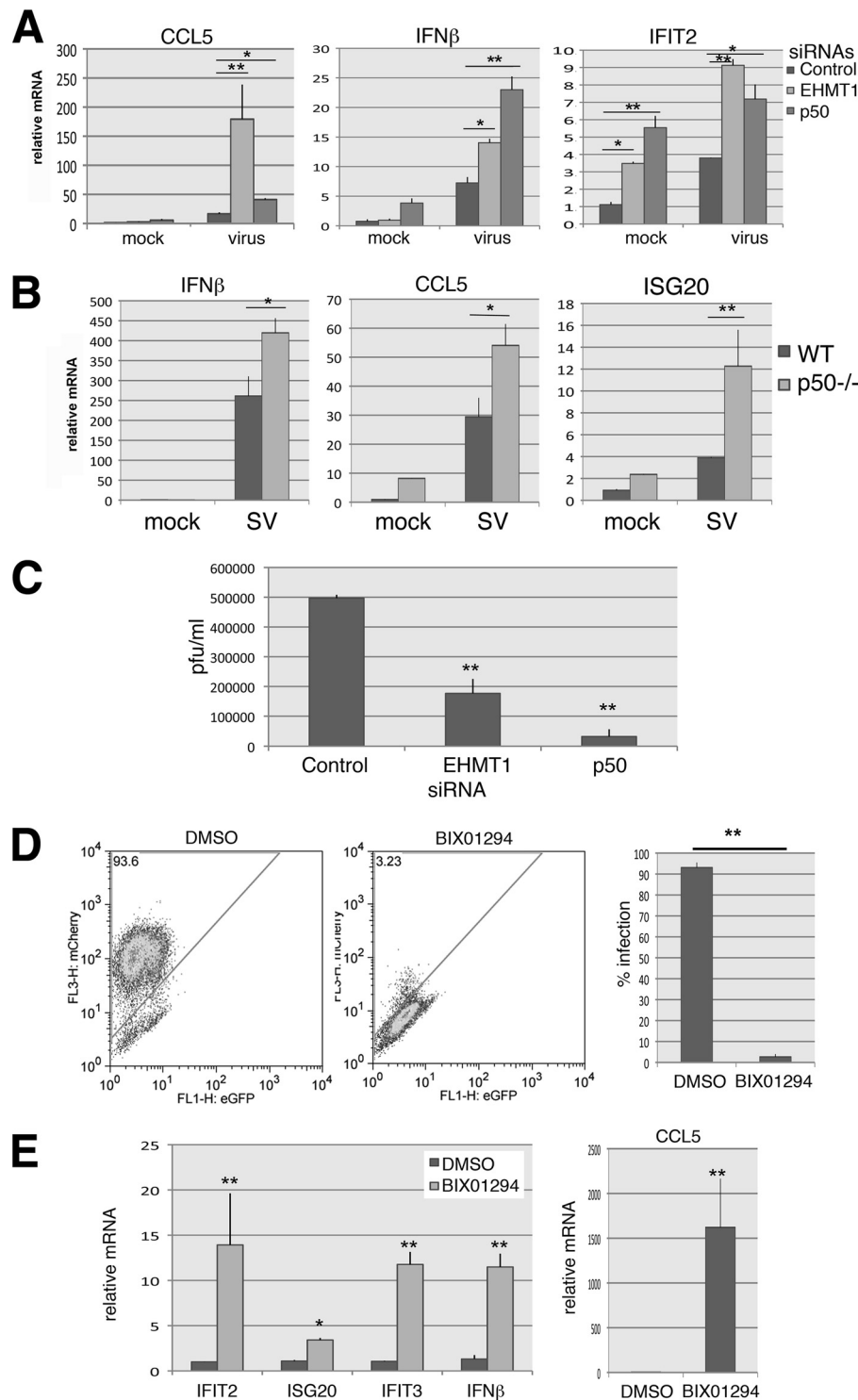


FIGURE 5. EHMT1- and p50-deficient cells are resistant to viral infection. *A*, HEK293-PB1 cells transfected with control siRNA or EHMT1-specific siRNA were infected with influenza virus at a m.o.i. of 0.5 for 18 h. The expression of *IFN β* , *CCL5*, and *IFIT2* was determined by RT-PCR, *, $p < 0.05$; **, $p < 0.01$. *B*, wild-type or p50-deficient MEF cells were infected with Sendai virus for 18 h. The expression of *CCL5*, *IFN β* , and *ISG20* was measured by RT-PCR, *, $p < 0.05$; **, $p < 0.01$. *C*, the culture supernatants from *A* were collected and were used to infect A549-PB1 cells. Twelve hours later, mCherry-positive cells were sorted by FACS (**, $p < 0.01$). *D*, A549-PB1 cells were treated with dimethyl sulfoxide (DMSO) or $5 \mu\text{M}$ BIX01294 for 48 h. The cells were then infected with influenza virus at a m.o.i. of 1.0 for 24 h. mCherry-positive cells were sorted by FACS. Chart represents quantification of three independent biological samples (mean \pm S.D.) (**, $p < 0.01$). *E*, A549-PB1 cells were treated as in *D*. The basal levels of *IFN β* , *CCL5*, *IFIT2*, *IFIT3*, and *ISG20* were measured by RT-PCR. *A*, *B*, and *E*, error bars represent the variation range of duplicate experiments (*, $p < 0.05$; **, $p < 0.01$).

expression patterns. Our study indicates that EHMT1 is critical not only for silencing the expression of NF- κ B-regulated genes and type I interferon-responding genes, but also for establish-

ing the signal specificity of gene expression. It has been elegantly shown that different immunostimuli induce different gene expression patterns (35). For example, PAM induces an

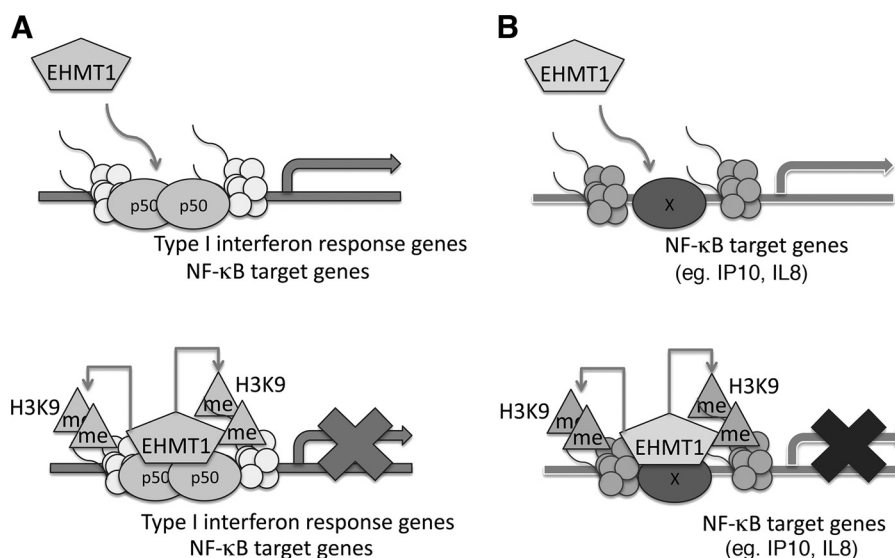


FIGURE 6. **Models of gene repression by EHMT1.** P50 (A) or factor X (B) recruits EHMT1 to inactivate the promoters via H3K9 methylation.

“inflammatory-like” program, whereas poly(I:C) induces an “anti-viral” program, and LPS induces both the inflammatory-like and the anti-viral programs (35). Under normal conditions, the expression of *IFNβ* is induced specifically by a few NF-κB agonists, such as LPS and poly(I:C) but not TNFα and IL1β. In the absence of EHMT1, the expression of *IFNβ* is induced by treating the cells with TNFα or IL1β, suggesting that EHMT1 is required for establishing the signal-specific induction of *IFNβ* (Fig. 4B and data not shown). Another consequence of using EHMT1 to maintain and establish signal-specific gene expression is that the capacity of gene induction is limited. For example, in response to TNF stimulation, the expression of *IL8* (3-fold) and *IP10* (2-fold) is higher in cells without the negative regulation of EHMT1 (Fig. 1A).

Our results show that EHMT1 is a negative regulator of type I interferon pathway (Figs. 4 and 5) and thus might be a potential new target for developing new antiviral drugs. Indeed, pharmacological inhibition of EHMT1 with BIX01294 protects cells from virus infection (Fig. 5D). However, EHMT1 has been implicated in playing a role in control of cognition and adaptive behavior in adult mice (36). Conditional EHMT1 and EHMT2 gene inactivation in postnatal mouse neurons led to defects in learning, motivation, and environmental adaptation (36), suggesting that long term inhibition of EHMT1 might affect proper brain functions. Furthermore, EHMT2 has been shown to be involved in cocaine-induced plasticity, and treating mice with BIX01294 increased the preference for cocaine (37). Thus, the potential of using BIX01294 as an antiviral drug might be compromised by adverse side effects related to the biological roles of EHMT1 in other systems such as the CNS. Developing an inhibitor that inhibits the binding between EHMT1 and p50 rather than the enzymatic activity of EHMT1 might be better a strategy but would be very difficult. Further experimentation using animal models will be needed to examine the clinical application of EHMT1 inhibitors.

In preparation of our manuscript, Fang *et al.* identified EHMT2 (G9a) as a negative regulator of the type I interferon response (38). It has been shown that EHMT1 and EHMT2

form a homodimer and heterodimer *in vivo*, and deficiency of either EHMT1 or EHMT2 in ES cells results in global reduction of H3K9me2 (39). Therefore, it seems that the EHMT1/EHMT2 heterodimer is the functional H3K9 methyltransferase *in vivo*. Our results indicate that EHMT2 is dispensable for the NF-κB pathway (Fig. 1A), whereas both EHMT1 and EHMT2 are required for repressing type I interferon pathway (Figs. 4 and 5 and Ref. 38).

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